

# Lack of N Regions in Fetal and Neonatal Mouse Immunoglobulin V-D-J Junctional Sequences

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## Summary

Much of T and B lymphocyte receptor diversity derives from the addition of nontemplated N regions at the junctions of receptor gene elements, although fetal T cells expressing  $\gamma/\delta$  receptors lack N regions. I have sequenced immunoglobulin H chain variable regions of PCR-amplified DNA and cDNA from fetal and newborn mouse liver and spleen cells. These sequences showed an absence of N regions. Only 1/87 DNA sequences and 17/146 RNA sequences contained N regions, in striking contrast to adult Ig sequences. These data show that N region insertion is a developmentally regulated process in B cells as well as in T cells, and demonstrate that receptor diversity in neonatal B cells is limited by the absence of N regions as well as by biased usage of Vh genes.

The initial diversity in Igs is afforded by the large number of V, D, and J gene elements used to create the L and H chain variable regions (1-5). In addition, there is considerably more diversity generated at the junctions of these gene segments. This junctional diversity is created by two mechanisms: (a) deletion of a variable number of nucleotides from the ends of the coding segments, presumably by exonuclease activity; and (b) subsequently, addition of a variable number of nucleotides to the V-D and D-J junctions of the H chain before ligation of the DNA (6). These latter nucleotides, called N regions, are nontemplated and are thought to be added by the enzyme terminal deoxynucleotidyl transferase (TdT)<sup>1</sup> (7-10). Junctional diversity increases the antibody repertoire by several orders of magnitude. The N regions and all of the D region are in CDR 3 and thus contribute significantly to the antigen-binding site.

Rearrangement of Ig coding elements in B cell precursors is a highly regulated process (11). At the Ig H chain (IgH) locus, D to J rearrangements (with possible N region addition at the D-J junctions) precede V to D-J rearrangement (again with possible N region addition at the V-D junction) during pro-B cell development. After successful IgH rearrangement, L chain V to J rearrangement takes place at the pre-B cell stage. L chain V-J junctions do not contain N regions, which correlates with the absence of TdT in most pre-B cells (12).

Other features of Ig rearrangement show developmental regulation; e.g., B cells generated during fetal and neonatal life overexpress Jh-proximal Vh genes (13-15). In contrast,

adult B cells show random Vh utilization (15-19), although one recent study suggested that IgH rearrangement may be biased towards the Jh-proximal Vh genes throughout life (20).

Developmental regulation of N region addition at the IgH locus has not been observed in the B cell lineage, but it is intriguing that fetal T lymphocytes expressing TCR- $\gamma/\delta$ , and some of their adult progeny, lack N regions (21-23). Extrapolating this observation to other lymphocytes is not obvious, however, since these  $\gamma/\delta$  T cells express only one V $\gamma$  and one V $\delta$  gene at each stage of their differentiation suggesting stringent selective pressure. The minor  $\gamma/\delta$  T cell subset shares many attributes with the minor B cell subset expressing the Ly-1 (CD5) marker (24). Both arise during fetal life, and become a minor component in most adult lymphoid tissue, except for specific sites of localization such as the skin, intestinal, and mucosal epithelia for  $\gamma/\delta$  T cells, and the peritoneal cavity for Ly-1 B cells (22, 23, 25-27). Ly-1 B cells have also been proposed to differ from conventional B cells in that they fail to undergo somatic hypermutation after antigen exposure (28, 29). These observations led me to investigate whether N region addition might be developmentally regulated in all or a subset of B cell precursors.

To examine this question, I analyzed IgH junctional sequences derived from PCR-amplified cDNA and genomic DNA from newborn spleen and liver, and DNA from day 17 fetal liver. These sequences were compared with those derived from PCR-amplified cDNA and genomic DNA of adult mice. The results show that N region nucleotides were lacking in almost all fetal and newborn junctional sequences derived from DNA, and from 90% of the newborn sequences derived from RNA. In contrast, N regions were found in most adult sequences. The addition of N regions thus appears to

<sup>1</sup> Abbreviations used in this paper: A-MuLV, Abelson-MuLV; RSS, recombination signal sequence; TdT, terminal deoxynucleotidyl transferase.

be a developmentally regulated process in progenitors of all murine B lymphocytes.

Several other findings were apparent in the large collection of Ig sequences generated in these studies. Overutilization of certain D and J gene elements was observed in both neonatal and adult Ig sequences. D regions showed preferential use of one reading frame, and D-D joins were rare. Non-random usage of D and J gene segments will restrict the size of the potential repertoire of adult as well as fetal/neonatal B cells. In addition, the diversity of fetal/neonatal B cells will be even more restricted both by the lack of N regions demonstrated here as well as the previously described preferential use of the Jh-proximal Vh genes (13–15). Developmental regulation of Ig gene rearrangement thus has a substantial impact on the capacity of the neonatal immune system to recognize the antigenic universe.

## Materials and Methods

**Source of Cells.** BALB/c mice were bred at the Medical Biology Institute (La Jolla, CA). Fetal livers were obtained from timed pregnancies, with day 0 being the day that a vaginal plug was observed. The newborn mice were used within 24 h of birth. All mice from a litter were pooled for the RNA samples. For the DNA samples, mice from two different litters were pooled. The adult RNA sequences were derived from four different spleen preparations from BALB/cBy mice that were 4–6 wk old. The adult spleen cells were cultured with 50  $\mu$ g/ml LPS for 3 d. Genomic DNA was obtained from individual spleens of two BALB/c mice, which were  $\sim$ 3 mo old.

**DNA, RNA, and cDNA Preparation.** Single cell suspensions of newborn spleen or liver were washed in PBS and then resuspended to  $2 \times 10^7$ /ml in PBS. 4 vol of water were added per 1 vol of PBS, and the cells were frozen and thawed three times then boiled for 10 min. 10  $\mu$ g of RNase was added, and the mixture was further incubated at 37°C for 1 h, then heated to 95°C for 10 min. 25  $\mu$ l of this DNA preparation was then added to the PCR reaction.

Adult genomic DNA was prepared by resuspending  $3\text{--}6 \times 10^7$  cells in 3 ml of cold LST (20 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>), to which 1 ml of cold 5% sucrose plus 4% NP-40 in LST was added. The nuclei were pelleted and resuspended in 1 ml cold LST to which 150  $\mu$ l of 0.5 M EDTA, 150  $\mu$ l of 5 M NaCl, 75  $\mu$ l of 10% SDS, and 10  $\mu$ l of proteinase K was added. The mixture was incubated at 37°C overnight, and then was phenol/chloroform extracted and dialyzed.

Total cellular RNA was extracted from single cell suspensions of newborn liver or spleen, or from the LPS-stimulated adult spleen cells, by the guanidinium thiocyanate/cesium chloride method (30), and cDNA was prepared with a C $\mu$  primer (5'-GGGGAATTCATTGGGAAGGACTGACT) as previously described (31).

**PCR Primers.** All oligonucleotide primers were made by Genosys Biotechnologies, Inc. (The Woodlands, TX). One primer for amplifying VhS107 (S107-1, 5'-GGTGAAGCTTGTTGGAATCTGGAGGA) starts at the third nucleotide of the S107 V1 gene. In practice, this primer was sufficiently homologous to amplify not only VhS107 genes, but also two J606 genes, a Vh10 gene, and several Vh7183 genes. Recently, a second VhS107 primer (S107-2) has been used that starts at nucleotide No. 22 from the start of VhS107 (5'-GGAGGAAGCTTGGTACAGCCTGGG), and this primer is much more VhS107 family specific. Each 5' primer had one change from the germline sequence to create a HindIII site.

The 3' PCR primer for cDNA preparations was the same primer used for cDNA synthesis. For newborn genomic DNA, a set of Jh primers was used: Jh1+4 (5'-GGGGAATTCCTGAGGAGACGGTGA), Jh2 (5'-GGGGAATTCCTGAGGAGACTGTGA), and Jh3 (5'-GGGGAATTCCTGCAGAGACAGTGA). All 3' primers had two to three changes from the germline sequence to create an EcoRI site. For PCR reactions 57 and 61 of the adult LPS-stimulated cDNA and for the adult genomic DNA, only Jh1+4 was used as the 3' primer.

**PCR Amplification and Sequencing.** 5  $\mu$ l of the cDNA or 25  $\mu$ l of the DNA was amplified in an Ericomp thermal cycler using the Gene-Amp kit (Cetus Corp., Berkeley, CA). Primers were used at 0.4  $\mu$ M. 25 cycles of amplification were performed with cycles of 1 min at 94°C, 20–40 s at 55–62°C, and 1–2 min at 72°C. The amplified DNA was chloroform extracted, ethanol precipitated, and digested with EcoRI and HindIII. The digested DNA was run on a 1.3% low melting point agarose gel, and the band (located at 360–390 bp) was cut out of the gel. For PCR-amplified cDNA, the agarose band was melted at 65°C, and was cloned and sequenced as previously described (31). For genomic DNA, we primed both the desired rearranged fragments and a lower molecular weight fragment that was primed by a relatively weak homology of the Jh primer with a sequence 220 bp into the VhS107 sequence. Therefore, the agarose band containing the appropriate sized undigested DNA from the primary PCR was melted, and 5  $\mu$ l of that was used in a secondary PCR. The secondary PCR was performed either with the original primers, or with the internal Vh primer, S107-2. However, the use of the same primer set for both amplifications led to some non-Ig sequences as a result of inappropriate priming. Thus, most of the genomic DNA sequences were derived from the nested primer set, and therefore predominantly use VhS107 genes. The size-selected amplified DNA from the secondary PCR was then processed as described above.

**Analysis of Junctional Sequences.** Since it is necessary to know the genomic sequence of the V, D, and J gene segments up to the heptamer of the recombination signal sequence (RSS) in order to unambiguously assess N region addition, I used a Vh primer homologous to a fully sequenced Vh family (32). Surprisingly, in the 254 VhS107 family sequences that I have obtained so far, only two of the three reportedly functional VhS107 genes were observed. This is even true at the DNA level, demonstrating that the V13 gene is not even used in nonfunctional rearrangements. Since the sequence 3' of the coding region, the RSS, is identical to that of V11, the reason for the absence of V13 is not known.

The VhS107 primer that we used for our original studies (31), S107-1, was sufficiently homologous to prime not only VhS107 sequences but also several Vh7183 and VhJ606 genes, and a Vh10 gene. Some of the latter are cloned and thus the sequence up to the heptamer is known (references 13, 33; Hartman, A., personal communication for Vh69.1; Hood, L., and S. Crews, personal communication for J60614a). For sequences using uncloned Vh genes, the Vh-D junction cannot be reliably assessed, and only those sequences that delete enough nucleotides to encroach upon the consensus Vh sequence are analyzable for N region nucleotides. However, the few nucleotides listed at the end of the uncloned Vh sequences in the neonatal sequences are consistent in most cases with our estimate of the continuation of the corresponding Vh sequence from the combined analysis of all adult and neonatal sequences. In our analysis, any junctional nucleotides that could be encoded by P regions (23) were counted as such. The Vh sequences routinely extend into CDR2. Sequences are grouped as deriving from the same germline Vh gene if they have two or fewer nucleotide differences from the prototypic sequence. Often there are dupli-

NEWBORN SPLEEN DNA

	V <sub>H</sub>	V or D	N	D	D or J	N	J <sub>H</sub>	#Deleted
#107:V1	GCA AGA GAT GCA TG							
92-3K	---	T GC					3	9-12
92-3R	---	A TG		A TGG TTA C	TA C		4	4-7
92-4A	---	I		AC GGT AG	T		3	4-5
92-4B	---	AT		TAC TAC GGT	AG		1	0
92-4E	---	AT G		AT TAC GAC	G		4	10-11
92-4H	---		P	TAT GA	T GGT T		3	4-9
92-4J	---			AT			4	1
92-4L	---			C TAC TAT GGT AAC	TAT		4	1-4
92-4M	---	AT		TAC TAC GGT AGT	AGC TAC		1	0-6
92-4N	---						1	8
92-4AA	---			G GTA AC	T		4	3-4
92-4AB	---	I		AT GGT A	A		4	8-9
92-4AC	---	AT G		AT GGT TAC TAC	G		4	10-11
92-4AF	---	AT G		AT TAC GAC	G		3	5-6
92-4AI	---	AT G		AT TAC G	AC		4	5-7
92-4AJ	---			TT TAC TAC GGT AGT AGC T	A		4	2-3
92-4AK	---	AT		AGG TAC G	AC		4	5-7
92-4AL	---	A		C TAC GGC	TAC		4	4-7
92-4AN	---	AT		TAC TAC GGT AGT AGC TAC G	T		4	9-10
92-4AQ	---			A TTA CTA CCG CTA	C		3	3-4
92-4AR	---	T		ACT ATG GTA A	CT AC		1	2-6
#107:V11	GCA AGA GAT ATA							
92-3I	---	A		C TAT GGT AAC TAC G			4	5
92-4D	---	T A		CT ACG GTA GTA G	CT A		4	6-9
92-4F	---	AT		TAC TAC GGT AGT AG	C TA		4	6-9
92-4P	---	AT		GGT AAC TAC G			4	3
92-4Q	---	AT		GAT GG	T TAC TA		4	3-9
92-4AE	---	AT A		GG TAC G	AC		2	2-4
92-4AG	---	TA		TGG	TA		1	3-5
V811	ATG AGA ??							
92-4R	---			C TAC TAT GAT TAC G	AC		4	5-7
J606:87-1X	ACC AGG ??							
92-3G	---			TAT GGT AAC TAC			4	4
7183:82-1E	GCA AGA ??							
92-3B	---	G		AT GGT AAC	TAC		4	4-7
92-3F	---	GAT C		TT ACT ACG GTA GTA	G		4	10-11
7183:81-1A	GCA AGA ??							
92-3D	---	C		AT GGT AAC TA	C		3	3-4
92-3O	---			GTA GTA G			4	3
92-3P	---	C		AT G	AT TAC		4	2-7

Figure 1. Sequences of Vh-D-Jh junctional regions of spleen DNA from newborn mice. Sequences are grouped according to Vh usage, with the Vh family listed first followed by the name of the cloned gene or of a prototype sequence. For all of the sequences above the line, the germline Vh gene is cloned and sequenced up to the heptamer of the RSS. For sequences below the line, the sequence of the germline Vh genes up to the heptamer is not known. In the latter cases, nucleotides are listed as N nucleotides only when the Vh sequence ended before the consensus coding sequence shown. P region nucleotides are underlined. In the majority of cases, some junctional nucleotides could be encoded by either V or D, or either D or J (including P region dinucleotides as a continuation of the germline sequence). Nucleotides that could be encoded by either of two adjacent gene segments are listed in the "V or D" or "D or J" columns. D region sequences are compared with the published sequences (3, 4) or to the two new potential D region sequences described in Results. The columns at the right indicate the Jh usage and the number of nucleotides that have been deleted from the Jh sequence, including the two P nucleotides as the start of the germline Jh sequence.

cate sequences derived from genomic DNA. Since the chance of finding duplicates due to the PCR amplification far exceed the chance of duplicate rearrangements with the target DNA put into an individual PCR reaction, all duplicate sequences from any one PCR reaction are discounted.

Results

*Analysis of IgH Junctional Sequences from Genomic DNA.* Junctional sequences of the rearranged IgH loci derived from PCR-amplified genomic DNA from spleen and liver of mice <24 h old are shown in Figs. 1 and 2, respectively. It can be seen that only one N region nucleotide was present out of 68 sequences analyzed, demonstrating that N regions are almost totally absent from newborn junctional sequences. No N region nucleotides were observed in the 19 sequences derived from day 17 fetal liver DNA (Fig. 3).

This lack of N regions is in striking contrast to the presence of N regions in the majority of published IgH sequences from adult mice (34). Since many of these sequences were

derived from responses to a limited number of antigens, preferential selection for antibodies with N region additions might have occurred. I thought it important to confirm the high frequency of N regions in PCR-amplified DNA from adult spleen. Accordingly, genomic DNA was prepared from adult spleen cells, and the junctional sequences were analyzed. The sequences derived from this PCR-amplified DNA represent a random collection of the rearranged IgH alleles present in adult spleen. Figs. 3 and 4 show that 83% of adult Ig have N regions sequences in at least one of their two junctions. 71% of the sequences have N regions at the V-D junction, and 56% of the sequences have N regions at the D-J junctions. These data confirm the previous results based on antigen-specific responses, and show that there is a dramatic difference in N region utilization between neonatal and adult IgH junctional sequences.

*Analysis of IgH Junctional Sequences from RNA.* Although most junctional sequences from newborn genomic DNA are lacking N regions, it is possible that the expressed functional Ig repertoire contains significantly more N regions. My se-

NEWBORN LIVER DNA

	V <sub>H</sub>	V or D	N	D	D or J	N	J <sub>H</sub>	#Deleted
<b>S107; V1</b>	GCA AGA GAT GCA TG							
92-2A	---	C		T ATG	AT		4	2-4
92-2C	---	AT G		GT AAC TA			3	9
92-2D	---	AT G		G	T TAC TA		4	3-9
92-2E	---	GCA		ACT G	GG		3	5-7
92-2G	---			G			4	2
92-2H	---	AT		TAC TAC GGT AGT A	G		4	5-6
92-2I	---			C TAC TAT AGG TAC G T	AC		4	5-7
92-2K	---	T		AC TAT GGT A			3	2
92-2M	---	GAT		GGT	TAC		1	4-7
92-2Q	---			T TAC TAC GGT AGT AG	C TA		4	6-9
92-2AB	---			T TAC TAC GGT AGT AGC	TAC		4	4-7
92-2AD	---	AT		AGG	TAC		4	4-7
92-2AE	---	T		AT GGT A	AC TAC		2	2-7
92-2AG	---	A		C TAT GGT TAC GAC			4	4
92-2AI	---	GA		C AGC TCG GGC	TAC		4	4-7
92-2AL	---	AT G		GT A	AC TA		4	5-9
92-2AN	---	A		G TAT GGT A	AC TA		4	5-9
92-2AO	---			GG	T		2	13-14
92-2AP	---	C		T GG			4	6
92-2AR	---	AT		AGG TAC G	AC		4	5-7
<b>S107; V11</b>	GCA AGA GAT ATA							
92-2B	---	A		C TAT AGG TAC			4	4
92-2F	---	AT		GGT TAC G	AC		4	5-7
92-2O	---	GAT		GG	T TAC TA		4	3-9
92-2AJ	---	AT		GGT A	AC TA		4	5-9
92-2AK	---	AT		GAT TAC G	AC		4	5-7
92-2AM	---	A		C TAT GGT AAC TAC GT			3	12
<b>J606; 14A</b>	ACC AGG CC							
92-2AH	---			ATC TAC TAT GAT TAC GAC			4	4
<b>VH11</b>	ATG AGA ??							
92-2AA	---	TA		ATG ATT ACG AC	G		3	10-11
<b>VH10</b>	GTG AGA ??							
92-1J	---	C		AT GGT AAC	TA		4	1-3
92-1P	---	C		AT GAT GG	T		4	3-4
<b>7193, Misc.</b>	GCA AGA ??							
92-1H	---	G		AT GGT A	AC TA		4	5-9
92-1K	---	C		AC TG			4	9
92-1L	---	C		AT GGT AAC	TAC		4	4-7

Figure 2. Sequences of Vh-D-Jh junctional regions of DNA from liver of newborn mice. Sequences are displayed as described in the legend to Fig. 1.

quences from genomic DNA would have equally sampled all productive and nonproductive rearrangements from both pre-B and B cells. The analysis of RNA sequences will preferentially sample productively rearranged IgH alleles from activated B cells (18, 35-38). If antigens or idiotypic interactions (39) more often activate cells whose IgH receptors contain N regions, one would expect a higher percentage of sequences to contain N regions in the population of mRNAs than in the DNA analysis.

RNA was prepared directly from newborn spleen and liver, converted to cDNA, and amplified. Use of the C $\mu$  primer restricts the sample to primary B cells that have not under-

gone isotype switching. All of the sequences are summarized in Fig. 3 and Table 1, and Figs. 5 and 6 show a partial listing of the IgH junctional sequences derived from RNA, including all sequences with N regions. 11% of newborn spleen sequences and 9% of newborn liver sequences show N regions, most of which consist of only one or two nucleotides. Analysis of only the sequences using VhS107 genes shows an even larger difference: 1.4% of the newborn DNA sequences contained N regions, while 16% and 6% of sequences derived from newborn spleen and liver RNA, respectively, contained N regions. The increased presence of N regions in RNA sequences compared to DNA sequences suggests preferential

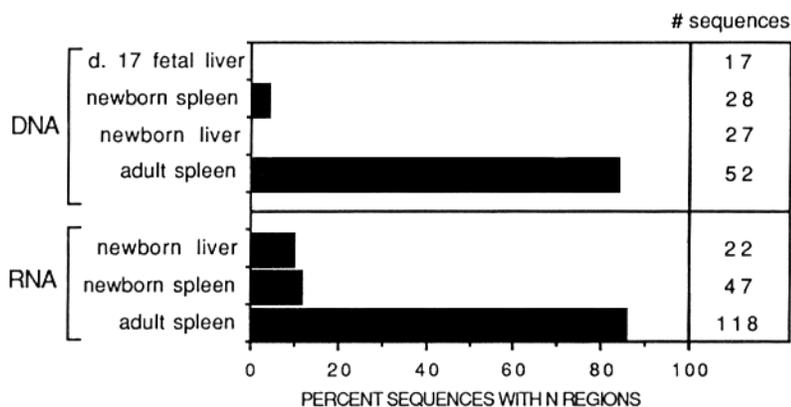


Figure 3. Percentage of IgH sequences that had any N region nucleotides (i.e., at the V/D junction, D/J junction, or at both junctions). Only sequences that used Vh genes cloned at the genomic level, and therefore whose Vh sequence was known up to the heptamer of the RSS, were used for this analysis. The source of DNA or RNA is shown on the left, and the number of sequences analyzed for each tissue is shown on the right.

ADULT SPLEEN DNA

	V <sub>H</sub>	V of D	N	D	D of J	J <sub>H</sub>	#Deleted
<b>S107:V1</b>	GCA AGA GAT GCA IG						
43-11A	---		T	TAC TAC GGT AGT AG	CT		1 5-7
64-11L	---		G GGC CC	T TAC GGT	TAC		1 3-6
64-11J	---		G	AT TAC TAC GGT AG		GG	1 4
64-11BC	---		GG GGG	TAC TAC GGT AGT AGC TAC G		G	4 6
64-11CI	---		T CTC	TAT TAC TAC GGT AGT		CGG G	1 4
64-11DG	---		TT	TAT GAT TAC GAC G		GG	1 4
69-1L	---		TT CT	T ATG ATT ACG		GGG GG	4 15
69-1AA	---		GA	GTC CTA G		CC AGG	4 10
71-2D	---		AG	A TGA TGG T		AT AAG	4 10
71-2AA	---		G	TAT GAT TAC GAC	G		4 5-6
71-2AB	---		C CG	C TAC GGC TAC	G		4 10-11
71-6N	---		T	TC TAC TAT GGT TAC G	AC		1 4-6
71-6AL	---		C	TTA CTA CGG CTA C		GT GAG GG	4 9
43-11B	---		GG GGG	TTA CTA CG		T ACC CG	1 4
64-11B	---		GT	TAT GGT TAC GAC			1 4
64-11F	---		G	T TAT AGG TAC GAC G		GG	1 10
64-11BA	---		AG GGA TCC C	AT TAC TAC GGT AGT AG	C T		1 5-7
64-11AD	---		G GA	A GGT			1 2
64-11AJ	---		G	AT TAC TAC GGT AGT AGC TAC G		C	1 5
64-11AO	---		CCC T	TT ACT ACG GTA GT	A_GCT AC		1 0-6
64-11BX	---		TG CGC			A	4 2
64-11BZ2	---		CG	TT ACT A		A	1 7
69-1AC	---		GAG	ATT ACT ACG GTA GTA G	C		4 6
71-3G	---		G	C TGG GAC G		AG GC	2 3
69-1AF	---	AT		TAC TAC GGT		CAC	4 8
71-6L	---	AT		TAC TAC GGT AGT AG		T	2 8
71-6P	---		G	A TTA CTA CGG CTA C		AC AGT TCG G	2 7
43-11C	---	AT		TAC TAC GGT AGT	AGC TAC		1 0-6
64-11BD	---	AT G		AT TAC G	AC		1 4
64-11A2	---			A			1 4
64-11AW	---	AT		TAC TAC GGT AGT	AGC TAC		1 0-6
69-1AM	---	GAT G		GT TAC TAC G			1 7
69-3G	---	G					4 14
71-2AH	---	AT		TAC TAC GGT AGT	AGC TAC		1 0-6
<b>S107:V11</b>	GCA AGA GAT A TA						
9-1	---		CC GCT	CTG GG		G AGA G	1 10
64-11A	---		CC CT	T ATT AC		T GG	1 5
64-11BK	---			G TAT GGT AAC		GGT TCT C	1 13
69-1A	---		T	TC TAC TAT		CCC CCC TCG	1 7
69-1H	---		CGG GA	G GTA GTA GC	T		4 3
69-1K	---		AAG AGG	TAT GGT AAC	TAC		4 4
69-1AL	---			GA C		CC	4 1
71-2AF	---	GA		C AGC TCG GGC TAC		CTT	4 22
71-2AG	---		GG GGT C	CT ACG GTA GTA GCT		GG	4 12
71-2AL	---		C GG	T ACT ACG GT		C C	4 2
71-6A	---		CGG GA	G GTA GTA GC	T		4 3-4
71-6AG	---		GGG	GGT AAC TAC		CCC	4 4
71-6R	---		CG GG	T ACG GCT AC		G AGG GGG	4 13
69-1U	---		TCT A	AG TAA		ACG GGG G	4 10
71-2F	---	AT A		GG TAC			4 4
<b>7183:Vh37.1</b>	GCA AGA CAT G						
69-1F	---		AAA	GGA C			4 8
<b>7183:Vh69.1</b>	ACA AGA GAT A						
69-12	---		G AGG G	AT GGT AAC TAC GI		G GGG	4 10
<b>7183:81X</b>	GCA AGA CAT G						
64-11CP	---	T		ATG GTA ACT AC		G TCT TT	1 3
<b>7183:Misc.</b>	GCA AGA ??						
71-3M	---		G	AG GTA CG	A		4 1
69-1G	---	CAT GGG GAA ATC AC		T TAT TAC TAC GGT AGT AG		GC	4 8
69-1Q	---	GGG GGG		GAT TAC G		GA	4 10
69-1AI	---			GCC TAC TAT GGT AAC TAC			4 5
69-1Y	---		G ATG G	GG C		GG CCC C	4 2
64-11BQ	---	CCA G		TT TAT TAC TAC GGT AGT AGC		CCT C	1 4
69-12Z	---		C	TAA C		CG GG	1 5
71-6AC	---	TA		G GGA CG		G GGT	4 7
64-11BW	---		CGG GG	A CTA TAG GTA C		CC	
				CTG GG		GG	1 3
<b>J606:87-1I</b>	ACC AGG ??						
64-1AS	---		CT	C TAT GGT AAC TAC G			1 4
64-1AY	---		A TCC	ATC TAC TAT GGT AA	C TAC		1 2-6
64-11E	---		G	AG GTA CG			1 4
<b>J606:87-2AB</b>	ACC AGG ??						
64-11AI	---	GAG GGG G		TT ATT ACT ACG GTA GTA GCT ACG		GC	1 2
64-1AE	---		A TTT GAG ACC CT	T CTA TGA T		C	1 5
64-11AK	---			T ATG GTT AC		C CC	1 4
64-11DB	---		T T	CT ACG GTA GTA G		TG	1 5
<b>J606:87-2J</b>	ACC AGG ?						
64-11AB	---		A	GAT G	G		1 7-8
64-11AQ	---	AC		T GGG ACG T		C	1 5
64-11AZZ	---			T ATT ACT ACG GTA GTA GCT AC			1 9
<b>VH 10</b>	GTG AGA ??						
69-1J	---	G		AT GG	T TAC		4 3-7
69-1B	---	CAG GC		C TAC		CCC C	1 4
71-6AB	---	G		AC TGG G		GA GAG GG	4 2
<b>VH 11</b>	ATG AGA ??						
71-2AD	---			TAT GAT GGT TA	C TAC		1 2-6

Figure 4. Sequences of Vh-D-Jh junctional regions of DNA from 3-mo-old spleen cells. Sequences are displayed as described in the legend to Fig.

**Table 1.** Presence or Absence of N Regions in Adult vs. Newborn IgH Junctions Does Not Correlate with Vh Gene Usage

Vh Gene	Newborn spleen and liver sequences with N				Adult spleen + LPS sequences with N			
	V/D	D/J	Either	Total analyzed	V/D	D/J	Either	Total analyzed
			<i>n</i>				<i>n</i>	
Cloned								
S107; V1	2	0	2	36	25	17	26	31
S107; V11	4	2	4	17	46	45	58	70
7183*	0	0	0	14	7	5	7	7
J558	0	0	0	2	2	3	3	3
Other				0	5	6	6	7
Total	6	2	7	69	85	76	100	118
	9%‡	3%	10%		72%	64%	85%	
Uncloned								
7183, All	(2)	2	(4)	20		25		33
J606; 57-11	(2)	1	(3)	24		45		60
J606; 57-2AB	(1)	1	(2)	19		35		49
VH10	(0)	0	(0)	10		33		55
Other	(0)	1	(0)	4		5		7
Total	(5)	5	(9)	77		143		204
	(6%)‡	6%	(12%)			70%		

Sequences are from RNA of newborn cells or from adult spleen that was cultured with LPS. The number of V/D or D/J junctional sequences that contained N regions are shown, as well as the number of sequences that contained N regions in either of the two junctions. For the sequences using uncloned Vh genes, only nucleotides that are likely to be nongermline encoded at the V/D junction, as described in Figs. 1 and 2, are listed. Since this is an estimate of the number of N regions, those figures are listed in parentheses.

\* Cloned Vh7183 genes that were observed are Vh37.1, Vh50.1., and Vh69.1.

‡ Figures represent the percent N regions.

activation of neonatal B cells expressing Ig receptors with N regions. However, 90% of the newborn IgH cDNA sequences are still lacking N region nucleotides, and therefore are very different from adult Ig sequences. No somatic mutation was observed in the RNA sequences presented here, since the rate of sequence divergence from known germline genes is 1/900 in this analysis, the same as the error rate due to PCR alone in our previous analysis of replicate clones from the same hybridomas (31).

The paucity of N regions in newborn antibodies was found not only for S107 genes but also for IgH sequences that use Vh7183 genes, a Vh family overrepresented in fetal and neonatal B cells, as well as for the Vh10 and VhJ606 genes which were homologous enough to the S107-1 primer to be amplified

(Table 1). Thus, the absence of N regions is not restricted to a particular Vh family.

To obtain comparable data from adult splenic RNA, I have generated >300 junctional sequences from cDNA of LPS-stimulated adult spleen cells, a sample of which is shown in Fig. 7. The few secreting B cells in the adult spleen contribute the majority of RNA (18), so the mitogen LPS, which is thought to randomly stimulate primary B cells (40, 41), was used to increase the level of IgM mRNA in all stimulated cells. Table 1 presents a comparison of the number of sequences containing N regions observed in the adult and neonatal RNA sequences. 85% of adult sequences had N region additions at either the V-D or D-J junctions, a figure almost identical to the percentage of N regions in sequences derived from

**Figure 5.** Sequences from Vh-D-Jh junctional regions of RNA from newborn spleen. Sequences are displayed as described in the legend to Fig. 1. This is a partial list of all of the sequences, and is nonrepresentative in that all sequences which have N regions are shown here. All of the sequences, including those not displayed here, are summarized in Fig. 3 and Table 1.

NEWBORN SPLEEN RNA

	V <sub>M</sub>	V or D	N	D	D or J	N	J <sub>2</sub>	#Deleted
#107:V1	GCA AGA GAT GCA TG							
76-1F	---	CA		GCT CGG GCT A	C		3	2-3
76-1BN	---			C			2	10
76-1CF	---			G			4	6
76-1CI	---		T	AAC TGG G	A		4	8-9
76-1CN	---	T		ACT ACG GTA GTA G			4	2
76-1CP	---	AT G		CT A	AC TA		4	5-9
76-1FG	---		CCGT	T TAC TAC GGT AGT AG	C T		2	6-8
76-1FJ	---	GAT G		GT TAC G	AC		4	5-7
87-2AE	---	AT G		AT TAC GA	C		3	3-4
87-2AG	---	AT		TAC TAC GGT AGT AGC T			2	7
87-2AR	---	AT G		GT AAC TA	C		3	3-4
88-1D	---	GAT G		GT TAC	TAC		4	4-7
88-1J	---	T		AT TAC TAC GGT AGT AG	CT		1	5-7
88-1R	---	AT G		AT GGT TAC TA	C		3	3-4
88-1AD	---	T G					2	9-11
88-1AJ	---	GA		C AGC TCG GGC TAC			2	6
88-1AO	---	A		A TTT ATT ACT ACG G			1	4
88-1AP	---			G TAT GGT A	AC T		2	5-8
#107:V11	GCA AGA GAT ATA							
76-1DK	---	AT		TAC TAC GGT AGT	AGC TAC		1	0-6
76-1E	---	T		T TAT TAC TAC GGT AG	T		2	9-10
76-1BP	---	A		C TGG	GAC		2	10
76-1CC	---	AT		TAC TAC GGT AGT AGC			2	10
76-1FM	---	GAT		GG	T		4	3-4
87-2AF	---	A		C TGG	GAC		2	10-13
88-1C	---		T	AAC TGG G	AC		1	4-6
88-1I	---		CT	AAC TGG			4	7
88-1P	---		GC	C TAC TAT AGG		A	3	2
88-1AC	---						2	0
7183:Vh37.1	GCA AGA CAT G							
76-1CD	---	AT G		AT GGT TA	C		3	3-4
76-1DN	---			C TAC TAT AGG TAC G	A		4	2-3
76-1DQ	---	A		C G	AC		4	5-7
76-1EE	---	AT		TAC TAC GGT AGT	AGC TAC		1	0-6
76-1FL	---	A		CT A	C		3	2-3
7183:Vh50.1	GCA AGA CAT G							
76-1BQ	---	A		C TAT AGG TAC GA	C		3	3-4
76-1CB	---	AT G		AT TAC GAC	G		3	10-11
76-1D	---			GG TAC G	AC		4	5-7
76-1BO	---	AT G		AT GGT	TA		4	7-9
76-1FA	---	A		C TAT A	GG TAC		1	7-12
76-1FB	---			GAC GT			3	2
7183:Vh69.1	ACA AGA GAT G							
76-1EJ	---	AT		TAC TAC GGT AGT AG	C T		3	3-4
76-1EH	---	AT G		AT TA			2	9
7183:57-2AC	GCA AGA ??							
76-1AM	---	C		AT GAT	TAC		1	3-6
76-1BE	---	CT		A ACT GGG A	C		3	11-12
76-1BG	---	CC		G GTA ACT A	C		4	11-12
7183:68-5W	GCA AGA ??							
76-1BI	---	GA		C TAT GAT			4	7
87-2AK	---	G		AT GAT TAC G		G	4	6
88-1AN	---			G			4	5
7183:Misc.	GCA AGA ??							
76-1CQ	---	C		AT GGT AA	C TAC		1	2-6
76-1AG	---			TAC TAT AGG TAC	G		4	10-11
76-1ED	---	GCA A		CT GGG AC			4	3
76-1AP	---		G	GGG			2	7
76-1ER	---			CTG GG		G	1	3
76-1AE	---	CAT		GAC			3	13
76-1BB	---		GG	AC TAT AGG TAC G	AC		4	5-7
76-1CK	---	C		AT GGT A	AC TA		4	5-9
87-2AH	---	C		AT TAT TAC TAC GGT AGT			3	10
76-1AN	---			CAT GAT TAC GAC	G		4	10-11
88-1AB	---			GGT AGT AGC TAC	G		3	2-3
J606:57-1I	ACC AGG ??							
76-1B	---			C			2	9
76-1H	---	AC		T ACG GCT AC		G	2	4
76-1AF	---		T	ATT TAT TAC TAC GGT AGT AGC T AC			2	11-13
76-1AR	---	C A		AC TGG	GAC		2	10-13
76-1BR	---	A		CG GTA G		C	2	12
76-1AI	---		T	AAC TGG GAC	G		3	10-11
76-1BM	---	G		TAT GGT AAC	TAC		4	4-7
76-1EM	---			TAT GGT AA	C TAC		1	2-6
76-1EP	---			GGT TAC GAC	G		4	10-11
87-2H	---	C		TAC GGT AG			2	0
87-2I	---	CC		A TGG	TT		2	7-9
87-2AB	---	C G		AC TGG GAC			4	4
J606:57-2AB	ACC AGG ??							
76-1CJ	---	C A		AC TG	G		2	0-1
76-1EO	---			ATT ACT ACG GTA GTA GC	T		2	9
76-2BQ	---	G		CAA CTG GGA CG		G	2	13
76-1FF	---			CTG GGA C			2	11
76-1DO	---	C		TAC GGT AGT AGC TAC	G		3	1-2
76-1EG	---		GC	T ACT ACG GC	TAC		3	9-12
76-1FC	---	C		GGT AGT AGC	T		3	7-8
76-1FP	---	T		ATG ATT ACG			3	10
76-1BA	---			TAT TAC TAC GGT AGT AGC	TAC		4	4-7
76-1CE	---			TAC TAT AGG TAC	G		4	10-11
87-2K	---			ATG ATT ACG AC	GT		2	0-2
VH 10	GTG AGA ???							
76-1DM	---			TAC G	AC		2	2-4
76-1BJ	---	G		AT GGT AAC TAC	G		4	10-11
76-1EA	---	CAT		TAC GG	C TAC		2	3-7
76-1EB	---			G			4	8
76-1EL	---			GAT GGT TA	C		1	2-3
76-1FH	---	C		AT TAC TAC GGT AGT AG	C T		1	5-7
87-2R	---	G		AT GGT AAC	TAC		4	4-7
87-2AI	---	CA		T ACT ATG G	TT		3	12-14

NEWBORN LIVER RNA

	V <sub>H</sub>	V or D	N	D	D or J	N	J <sub>H</sub>	#Deleted
<b>#107:V1</b>	GCA AGA GAT GCA TG							
87-3I	---			AC	JAT		4	0-2
87-3J	---	T		ATG ATT ACG	A		4	11-12
87-3P	---	AT		AGG TAC GA	C		3	1-2
87-3AE	---	AT G		AT GG	T TAC T		3	10-15
87-3AF	---	T G					2	7-9
87-3AJ	---	AT		TAC TAC GGT AGT AG	C TA		4	4-7
87-3AK	---	A		C TAT AGG CAC GAC	G		3	0
88-2C	---			C TAC TAT AGG TAC G	AC		4	3-5
88-2D	---	AT		TAC TAC GGT ACT AGC	TAC		4	2-5
88-2L	---	AT G		GT AAC	TAC		4	2-5
88-2AD	---	T		AC TAC GGT AGT AGC TA	CG		3	1-3
88-2S	---	GC		C TAC TAT GGT AAC	TAC		4	2-5
88-2V	---			G			4	1
<b>#107:V11</b>	GCA AGA GAT ATA							
87-3G	---	AT		GGT AAC	TA		4	0-1
87-3BH	---	AT		GGT A	AC TAC		2	0-5
88-2P	---	AT		GGT AAC	TA		4	0-1
88-2R	---			GGT		G	2	0
88-2N	---		CT	AAC TGG G	AC		2	0-2
88-2AG	---			C			3	0
88-2AN	---			GT ATG GTA AC	T		4	1-2
<b>7183:Vh50.1</b>	GCA AGA CAT G							
87-3AO	---			GAT TAC			4	2
<b>J558</b>	GCA AGA TC							
88-2AK	---			T TAC TAC GGT AGT	AGC TAC		1	0-4
<b>J606:57-1T</b>	ACC AGG ??							
87-3N	---	A		CT G	GG		3	3-5
87-3AH	---			GA			4	13
87-3AI	---	C A		AC TGG GAC	G		2	8-9
87-3BA	---			TAT TAC TAC GGT AGT AGC	TAC		4	2-5
87-3BC	---	A		CT ATG GTT ACG AC			4	1
87-3BJ	---	C A		AC TGG GAC	G		4	8-9
88-2T	---	G		TAT GGT AAC	TA		4	0-1
<b>J606:57-2AB</b>	ACC AGG ??							
87-3A	---			T TAC TAC GGT AGT	AGC TAC		1	0-4
87-3AA	---	CC		T TAC GGT AGT AGC	TAC		2	0-2
87-3BK	---	C A		A	C TG		3	1-4
<b>J606:57-1BA</b>	ACA TG??							
88-2AR	---			T GGT AAC TAC G		T	2	10

Figure 6. Sequences from Vh-D-Jh junctional regions of RNA from newborn liver. Sequences are displayed as described in the legend to Fig. 1.

adult DNA. The percentage of N regions at the two junctions was approximately equal, with 72% of V-D junctions and 68% of D-J junctions having N regions. The average number of N nucleotides per antibody was 4.8 for all adult antibodies containing N regions, whereas in newborn antibodies it was only 1.5 nucleotides. Of the junctions that had N regions, the average number of N nucleotides was approximately the same for V-D and D-J junctions. As with the newborn sequences derived from RNA, the percentage of N regions did not vary significantly among Vh families. Thus, the adult IgH junctional sequences predominantly contain N regions, and the N regions are longer than those seen in the newborn.

**New D Regions.** The availability of the germline sequences of the S107 Vh family and the D and Jh regions of the BALB/c strain of mouse was essential to the analysis of N region addition presented above. In analyzing the hundreds of sequences generated in this study, however, I have identified two additional sequences that are candidates for new germline D gene segments.

The first new sequence was similar to many DSP2 D gene segments and differed from DSP2.3 by only two nucleotides (3). 12 of the newborn sequences and 10 of the adult sequences contained identical core D region sequences of 9-11 nucleotides, including one that contained the whole putative D sequence (TCTACTATAGGTACGAC). Since it has been demonstrated that there are nine DSP2 genes (3, 42), and there are

only eight known sequences (3, 4), this sequence is likely to be the unsequenced DSP2 segment, and is referred to here as DSP2.10.

A second potential new D region sequence was observed. Four of the newborn sequences contained potential D or N regions of 10-13 nucleotides that were unlike any described D region and identical to each other in the core region. Analysis of all of the adult sequences revealed the presence of four additional sequences with at least nine nucleotides in common with this sequence. The consensus sequence is GACAGC-TCGGGCTAC. The last six nucleotides are identical to the 3' end of DFL16.2, but no other stretch of nucleotides is identical to any known D sequence in either conventional or inverted orientation. Since newborn sequences are almost totally lacking in N regions, the presence of this potential new D sequence in four newborn sequences as well as in the adult renders it unlikely that the sequence is a long N region coupled to DFL16.2. Thus, this is potentially a new D segment.

**P Regions.** Analysis of N region addition took into account the contribution of P regions to junctional diversity. P regions are dinucleotides palindromic to the two nucleotides of coding regions that are adjacent to the heptamer of the RSS. It has been proposed that P regions are appended to the coding region before deletion of nucleotides occurs (23). 152 V, D, or J region sequences from the adult LPS-stimulated spleen cells had not deleted any nucleotides from

RNA FROM 4-6 WEEK OLD SPLEEN + LPS

	V <sub>H</sub>	V or D	N	D	D or J	N	J <sub>H</sub>	#Deleted
#107:V1	GCA AGA GAT GCA TG							
61-1K	---			AT TAC G	AC		1	4-6
61-1N	---		CC	T CAT TAC TAC GGC		CAT	1	3
61-4AT	---		GGG	CGG CTA C	G		1	7-8
68-1D	---		G	GGT AA		A GG	1	5
72-1G	---		CC ACC TT	C TAT GGT AAC T		CC CC	1	2
68-1T	---	A		C TAT GGT TAC		CT	3	0
68-2F	---		C CC	T TAT TAC TAC GGT AGT AGC		CC	3	8
57-1AR	---		AG	C TAC TAT GGT A	A		4	8-9
57-2N	---		A	AT AGG			4	2
61-1V	---		G G	AC TGG G		G	4	8
61-4AT	---		GGG	CGG CTA C			1	5
61-4T	---		AA	ATT ACG ACG		GG	4	9
#107:V11	GCA AGA GAT ATA							
61-1AK	---		T	A TTA CGA C			1	7
61-4K	---		CC CCG	TGG G		G	2	3
61-4O	---	GAT		GGT T	AC TAC		1	11-16
61-4AH	---	AT		TAC TAC GGT AGT AGC		CC	2	2
72-1I	---			GG			2	5
72-2L	---		GGA G	TT ACT ACG GCT TAC G			2	4
72-1K	---		GA GCT	AAC TGG GAG		T	3	2
72-1R	---		CC C	TC TAC TAT AGG TAC GAC GT		T G	3	2
61-4X	---		G	TAT GAT TAC G		T	3	0
68-2D	---	TA		ACT A	C		3	13-14
79-1G	---		GGG	AC TAT AGG TAC GAC		GGG AA	1	6
61-1A	---			GGT AAC TAC		CCC TA	4	6
7183:Vh50.1	GCA AGA CAT G							
57-2BE	---		G ACA	TAC TAC GGT		CA	2	6
61-1S	---		CT	GGT AGT		C	4	5
61-1Z	---			G GCC TAC TAT GGT AAC TAC G			4	8
80-1L	---		GGA GAA GGG GGG	TTA CTA CG		A	4	10
7183:57-2AC	GCA AGA ??							
61-1E2	---	AAG GG		G TAT GGT AA	C TAC		1	2-6
57-2AG	---	CAG		ATT ACT ACG GC		A	2	4
57-2AC	---	CGG GAG		GGG AC		C CC	3	6
57-1BJ	---	AAC C		TT TAT TAC TAC GGT AGT AGC		CFC	4	13
68-5H	---	CGG CC		C TGG		A	4	8
J606:57-2AB	ACC AGG ??							
57-1AN	---		TGG T	CT ATG ATT ACG ACG		AGG GGC GA	1	8
57-2BB	---			AC TAC GGT AGT AGC TA	C		3	3-4
68-1C	---	CCC TCT		AAC TGG G	A		4	8-9
68-1A	---			TAC		AC	1	5
68-1K	---			ACT GGG ACG		GGG	3	10
68-2U	---	CCA		GGT T		TT	2	7
72-1F	---	TC		C GGT A		CC CCT	1	3
72-1H	---			GAT T		TT	2	4
72-2A	---		CC	A ATT TAT TAC TAC GGT AG		G GT	3	15
72-2I	---		AG CCG	ATT ACT ACG GTA GTA GCT		GAT	2	13
J606:57-1I	ACC AGG ??							
61-1AV	---			A ACT GGG ACG T		C	2	6
61-1AJ	---	G		TAT GGT AAC TAC		G	1	1
61-4B	---		G	T GGT TAC G		TT CCT GGA	3	10
61-4L	---	A		AAT GGT	T		2	7-8
61-4Z2	---		G A	GT AAC TAC GT		GGG	2	12
68-2N	---			ACG GTA		CCG GGG	3	10
68-5S	---	C		AT TAC TAC GGT AGT AG	C T		3	3-5
68-5T	---		CT	T TAC TAC GGT AGT AG		A TC	3	6
72-1O	---		CFC C	TT ACT ACG GC		CC	2	5
72-2J	---			GAC		AG	3	3
Vh 10	GTG AGA ??							
57-2AE	---		CAC GA	T TAC TAC GGT AGT		TG	1	2
61-4AL	---	CTC CGA GGGGGG		ACT GGG ACG		GAG	2	4
57-1H	---		A CGA	GGT AAC TAC		TTC G	3	2
68-1L	---	GAC CCT		TAT GGT AAC TAC G		GA	3	4
68-5F	---		C C	CT GGG ACG		GGA GGT	3	7
57-2P	---	CA		T GGG AC		C CFA T	4	2
57-1AC	---	CGA		GGT AGT AGC TAC			4	10
57-2AF	---	CGA		ACT		T	4	14
57-1AJ	---		T CAG CT	A ACT GGG			4	7
57-2BF	---			GAT GG	T TAC TA		4	3-9

Figure 7. Representative collection of sequences from Vh-D-Jh junctional regions of RNA from young adult spleen cells cultured with LPS. All sequences are summarized in Fig. 3 and Table 1. Sequences are displayed as described in the legend to Fig. 1.

the coding regions, and thus could be analyzed for the presence of P nucleotides. 63% of those sequences contained nucleotides compatible with P region utilization, thus confirming the generality of this proposed mechanism. Because the data presented here support the existence of P regions, any nucleotides that could have been encoded by this mechanism were interpreted as P rather than N regions.

Neonatal antibodies also had sequences that were compatible with being P regions. However, in many cases, the P nucleotides (as well as other junctional nucleotides) could

also have been encoded by the adjacent germline segment, making it impossible to assess the contribution of P nucleotides to neonatal antibodies. It is striking that >80% of the junctions from the neonates contain from one to five nucleotides that could have been encoded by either of the two adjacent coding segments, as previously observed in adult sequences that do not have N regions by Ichihara et al. (43). In the data presented here, the overlapping nucleotides are seen as often in both neonatal and adult junctional sequences that do not have N regions. However, since most adult sequences

**Table 2.** *Jh* Utilization

RNA source	Sequences analyzed	Percent Usage			
		Jh1	Jh2	Jh3	Jh4
	<i>n</i>				
Adult (4–6 wk)					
Spleen + LPS					
No. 1	53	15	23	49	13
No. 2	49	10	33	41	16
No. 3	59	3	24	49	24
No. 4	18	0	28	56	5
Total	179	8	26	47	18
Newborn spleen	113	15	30	21	34
Newborn liver	28	7	21	25	46

have N regions, and most neonatal sequences do not, it is only a prevalent observation in the newborn.

**Nonrandom Usage of D and J Elements and D Region Reading Frame.** The presence of such a large number of nonselected Ig junctional sequences permits the analysis of several other parameters that contribute to Ig diversity in addition to N regions. Any nonrandomness in the use of gene segments would restrict the size of the resulting repertoire. Determination of the relative frequency of usage of the four Jh genes is possible in sequences derived from cDNA, since the C $\mu$  primer should randomly amplify sequences using all Jh genes. Only cDNA sequences were used to avoid the potential problem that the Jh primers used in genomic DNA analysis might not be equally effective in priming. As shown in Table 2, the neonatal sequences used Jh4 predominantly, and the adult sequences used Jh3 most often. Four different adult spleen preparations were analyzed and yielded similar frequencies, indicating little mouse-to-mouse variation. All populations used Jh1 the least. For D regions, DFL16.1 is significantly overutilized in both neonatal and adult antibodies. DQ52 is also overrepresented in the adult. In the neonate, DQ52 and the new DSP2 gene, DSP2.10, are somewhat overutilized (Table 3). In addition, it can be seen that all D regions except DQ52 are used preferentially in one reading frame (Table 3).

**D-D Joins and Inverted D Regions.** Some D region sequences can be accounted for by the existence of D-D joins or by being inverted complements of D regions (44, 45), but the rate at which either of these structures is used in randomly selected B cells is not known. Out of the 397 adult RNA and DNA sequences presented here, only five sequences had potential D-D joins, each half of which consisted of at least five nucleotides in common with a known D region. This is a maximum estimate of D-D fusions, since potential D-D joins cannot be reliably distinguished from a single D region in addition to N nucleotides which fortuitously match part of another D region sequence. Two of these D-D joins con-

sist of an inverted D segment followed by a D region in normal orientation. In addition to these two potential inverted D regions, there are six D/N sequences with five to six nucleotides that are identical to the inverted complement of a D sequence.

In contrast to the adult sequences, none of the 233 sequences from fetal or newborn RNA or DNA contained inverted D region sequences or potential D-D joins. I conclude that D-D joins and inverted D regions are rare in the adult and absent in the neonate.

## Discussion

The data presented here clearly show that N regions are almost totally lacking in neonatal IgH sequences. The analysis of fetal and newborn DNA, which equally detects productive and nonproductive alleles of both pre-B cells and B cells, shows a virtual absence of N regions. RNA analysis predominantly assays transcripts of productive alleles of B cells and RNA from activated cells will be overrepresented (8, 35–38). Such analysis showed that 10% of the junctional sequences contained N regions, most of which consisted of only one or two nucleotides. In the VhS107 family, from which most of our DNA sequences are derived, the percentage of N regions increased from 1.4 to 16% when newborn DNA sequences were compared with those from newborn splenic RNA. Since RNA levels are raised in activated cells, the increase in N regions in RNA-derived sequences suggests that antigens or cellular interactions have preferentially activated B cells whose IgH sequences contain N regions. Alternatively, it is possible that there may be an advantage for IgH sequences with N regions in the transition from pre-B cell to B cell, perhaps in enhancing binding to surrogate L chains such as V<sub>preB</sub> or  $\lambda_5$  (46). The RNA analysis also included significant numbers of sequences from four different Vh families, and the lack of N regions is apparent in all. In contrast to the neonatal sequences, the sequences from adult DNA and RNA both show ~84% N regions, and the length of the N regions is significantly longer.

It has been shown that TdT levels rise slowly in the developing thymus (47, 48), which correlates with the absence of N regions in fetal TCR- $\gamma/\delta$ . Similarly, the absence of N regions in neonatal B cells may be associated with a relative absence of TdT in their precursors. In agreement with this hypothesis, TdT was not found in murine fetal liver in one study (48), whereas TdT is readily found in a small percentage of cells with B lineage markers in the adult bone marrow (12, 48). However, these data are in apparent contrast to the presence of TdT in a number of Abelson-MuLV (A-MuLV)-transformed cell lines derived from fetal liver (4, 7, 10). There are only a small number of fetal Ig sequences, mainly from these A-MuLV-transformed fetal liver cell lines, and a number of these sequences do have N region nucleotides (4, 7, 10). These later observations have led to the tacit assumption that most B cell precursors express TdT and therefore will show N region insertion. However, the absence of N regions in neonatal B cells that is shown here is likely to be associated with a relative absence of TdT in their precursors.

**Table 3.** *D Region Utilization and Reading Frames*

	RNA from LPS-stimulated adult spleen				Newborn RNA				Nonproductive allele of adult spleen DNA			
	I*	II	III	Total	I*	II	III	Total	I*	II	III	Total
All DSP2 (9 genes)	96	17	11	124	55	6	0	61	2	3	3	8
DSP2.3, 4, or 6	12	1	1	14	6	0	0	6	1	0	0	1
DSP2.2	6	10	2	18	8	3	0	11	0	0	0	0
DSP2.5, 7, or 8	38	3	1	42	19	2	0	21	0	1	1	2
DSP2.9	10	1	2	13	4	0	0	4	0	1	0	1
DSP2.10	17	0	1	18	14	0	0	14	1	1	2	4
DSP2 any	13	2	4	19	4	1	0	5	0	0	0	0
DFL16.1	43	8	3	54	26	7	0	33	4	4	0	8
DFL16.2	14	12	3	29	3	1	0	4	0	0	1	1
DFL16.1 or 2	5	1	2	8	0	0	0	0	0	1	1	2
Subtotal	158	38	19	215	84	14	0	98	6	8	5	19
DQ52	28	9	24	61	12	2	2	16	2	0	1	3
Total	186	47	43	276	96	16	2	114	8	8	6	22

The preferred reading frame (Frame I, nomenclature of reference 41) of all DSP2 and DFL16.1 genes is the tyrosine-rich reading frame. The preferred reading frame of DQ52 is C AAC TGG GAC. If the reading frame is one nucleotide back, it is called Frame II; Frame III is one nucleotide forward (41). The sequences used here are the ones in which a D region could be clearly identified. In the adult RNA sequences, for example, there are an additional 36 sequences with D regions of three or fewer nucleotides, and 10 sequences with longer but unidentifiable D regions.

\* Reading frame.

This apparent discrepancy between our results and data from the A-MuLV fetal liver lines has three potential explanations: (a) A-MuLV may not transform all fetal B cell precursors randomly; (b) the small number of previously available sequences is not an accurate representation of most fetal Ig sequences; and (c) the transformation process or the transition to in vitro culture may induce TdT in the fetal liver cells. This latter hypothesis is based on the observation that when normal fetal or neonatal thymocytes are placed in vitro overnight, the level of TdT production quickly rises to that of adult thymocytes (47).

The absence of N regions in newborn Ig junctions shown here suggests that TdT does not reach maximum levels until some time after birth in B cells, similar to the slow rise in thymocytes. This implies that the enzymatic components of the recombinational machinery are differentially regulated, with the recombinase activities appearing much earlier in ontogeny than TdT for both B cells and at least some T cells.

In addition to the striking observation of the absence of N regions in fetal and neonatal antibodies, several observations pertaining to D and J usage were also made. Most of the DSP2 genes and DFL16 genes were noted to preferentially use one reading frame (Table 3). Similar overuse of one D region reading frame has been observed in hybridomas and myelomas (43, 49). In those cases, however, it could be ar-

gued that, since the preferred reading frame encodes a tyrosine-rich D segment, the limited number of antigens used as immunogens for the majority of the current available sequences (34) might select D regions using that reading frame. That argument is supported by the observation that anti-GAT and anti-DNA antibodies preferentially use a different reading frame (50, 51). However, the collection of sequences presented here is a random sample from all of the B cells in the spleen or liver. Thus, the sequences presented here demonstrate that the primary repertoire of Ig preferentially uses DSP2 and DFL16 genes in one reading frame, and that antigen selection is probably not responsible for selecting the predominant frame.

Several examples of nonrandom usage of D and J gene elements were observed in both neonatal and adult sequences. The large number of sequences shown here extend previously published studies showing that Jh1 is significantly underutilized and that the more 3' Jh regions are overrepresented (4, 19). Nonrandom use of J segments has also been described for human Jh (52) and for mouse Jk segments (53).

DFL16.1 was observed to be overutilized in hybridomas and myelomas (43), and here, I show that it is also overutilized in this random collection of spleen sequences. The vast overutilization of DFL16.1 cannot easily be explained by antigenic selection, since all DSP2 and DFL16 genes are tyro-

sine rich and similar in amino acid sequence. Also, DQ52, as well as DFL16.1, was significantly overrepresented in the adult sequences, and both genes were overutilized in the sequences derived from the nonproductive allele of the adult DNA (Table 3), where antigen selection cannot occur. The prevalence of DQ52 may simply be its proximity to the Jh cluster, since it is located only 700 bp 5' of Jh. However, the predominance of the most 5' D segment, DFL16.1, cannot be explained by a similar argument, as it is located 78 kb from Jh1. The first step in H chain rearrangement is a D to Jh join. This initial join often involves a DQ52 gene (52, 54-56), and in one study DFL16.1 was also overutilized in initial joins (56). In murine A-MuLV lines, the initial D-Jh rearrangement is often deleted and replaced by a secondary D → Jh rearrangement, in which a more 5' D region, especially DFL16.1, joins to a more 3' Jh (56, 57). The prevalence of DFL16.1 and the 3' Jh segments that I observed is in agreement with prevalence of secondary D-J rearrangements. However, if secondary D-J rearrangements were the explanation for the overutilization of DFL16.1 and the 3' Jh genes, one would predict that there should be preferential association of Jh1 or Jh2 to DQ52 or the 3' DSP2 genes as examples of primary joins, and preferential association of DFL16.1 with Jh3 or Jh4 as examples of secondary joins. This was not observed. In fact, there was a slight bias in favor of preferential association of Jh1 and DFL16.1 and of Jh4 with the 3' DSP2 genes in the neonatal sequences. Thus, the explanation for the overutilization of these D gene segments appears to be neither antigen selection nor a preponderance of secondary D-J joins. An alternative explanation is that since the RSS sequences are different for the four J regions and for each

D family, and since the frequency of recombination depends upon the exact sequence of the RSS (58), it is possible that these sequence differences affect the rate of usage of the various D and J elements in the initial joins.

One important implication of the lack of N regions in neonatal antibodies is that the repertoire of neonatal antibodies will be different from that of adult antibodies. First, the lack of N regions early in ontogeny will lead to a preponderance of H chain CDR3 sequences that will only rarely be seen in the population of adult antibodies. Also, since the lack of N regions in neonatal B cells is not compensated for by deletion of fewer nucleotides from coding regions or longer D regions, the neonatal CDR3 regions are shorter than those of the adult. Thus, the neonatal antibody population, which not only is relatively devoid of N regions but also preferentially uses Jh-proximal Vh genes, would not be merely a subset of the adult antibodies, but to a large extent would be a different population, possibly with a different range of specificities.

As for T cells, B cell differentiation may occur in two or more distinct waves, with N region addition being associated only with the later waves. Both Ly1 B cells and  $\gamma/\delta$  T cells arise early in ontogeny, and become a minor population in the adult. Since a substantial percentage of newborn liver and spleen B cells are not Ly1 B cells (27), the data presented here extend beyond this subset of B cells and include most or all conventional B cells as well. The data suggest that fetal and neonatal B cells as well as  $\gamma/\delta$  T cells may represent vestiges of more primitive immune systems with limited receptor diversity.

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The excellent technical assistance of Donna Thuerlauf is gratefully appreciated. I thank Dr. Donald Mosier for a critical reading of the manuscript.

This work was supported by grant IM-513 from the American Cancer Society.

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Received for publication 13 June 1990 and revised form 20 August 1990.

*Note added in proof:* After this manuscript was submitted, a report showing that IgH junctions in the VhJ558 family have fewer N region nucleotides early in ontogeny, and that such junctions often have overlapping sequences, was published (59).

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